Impact of nitric oxide's bidirectional role on glaucoma: focus on Helicobacter pylori-related nitrosative stress

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Abstract: Nitric oxide (NO), a small molecule generated ubiquitously, targets a plethora of tissues to regulate both physiological and pathophysiological functions. NO overproduction, stimulated by microenvironmental conditions, is the main component that dysregulates the tight balance between its beneficial and damaging roles in ocular homeostasis. Considering the protective functions of NO against glaucoma, its endogenous release facilitates aqueous humor drainage and regulates ocular blood flow, maintaining a normal intraocular pressure. NO overproduction generates free radicals, such as peroxynitrite, which induce a vicious circle of vascular disharmony and dysregulation, transient ischemia, nitrosative stress, neuronal degeneration, and permanent glaucomatic injury. Helicobacter pylori (Hp) is considered a burdening factor of glaucoma. NO overproduction and possible systematic dispersion in Hp infection (Hp-I) could suggest a potential pathophysiological bridge between these conditions. In this review, we aim to elucidate the role of NO in glaucoma with respect to Hp-I, with the aim to stimulate further studies.

DOI: https://doi.org/10.1111/nyas.14253

Impact of Nitric Oxide's Bidirectional Role on Glaucoma: Focus on *Helicobacter Pylori* Related Nitrosative Stress

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ABSTRACT

Nitric oxide (NO), a small molecule being generated ubiquitously, targets a plethora of tissues to regulate both physiological and pathophysiological functions. NO overproduction, stimulated by microenvironmental conditions, is the main component that dysregulates the tight balance between its beneficial and damaging role in ocular homeostasis. Considering the protective functions of NO against glaucoma, its endogenous release facilitates aqueous humor drainage and regulates ocular blood flow maintaining a normal intraocular pressure. NO overproduction generates free radicals, such as peroxynitrite, that induce a vicious circle of vascular disharmony and dysregulation, transient ischemia, nitrosative stress, neuronal degeneration and permanent glaucomatic injury. *Helicobacter pylori (Hp)* is considered a burdening factor of glaucoma. NO overproduction and systematic possible dispersion in *Hp* infection (*Hp-I*) could suggest a potential pathophysiological bridge between these conditions. In this review, the authors aim to decode the role of NO in glaucoma in respect to the *Hp-I*, with the perspective to countenance and stimulate further studies.

RUNNING HEAD: The role of *Hp*-I-related NO in glaucoma

KEYWORDS: Nitric Oxide; NO; *Helicobacter pylori*; *Hp*; Glaucoma

ABBREVIATIONS: AD; Alzheimer's disease, AH; aqueous humor, BRB; blood–retinal barrier, CNS; central nervous system, cGMP; cyclic guanosine monophosphate, *Cag*A; cytotoxin-associated gene A; ET; endothelin, GBS; Guillain-Barré syndrome, *Hp; Helicobacter pylori, Hp-I*; *Helicobacter pylori* infection, HTM; human trabecular meshwork, IOP, intraocular pressure; NF-κB; nuclear factor kappa-light-chain enhancer of activated B cells, iNOS; inducible NOS, IOP; intraocular pressure, LPS;

lipopolysaccharide, NMDA; N-methyl-D-aspartate type, L-NAME; N-nitro-L-arginine methylester, NO; Nitric Oxide, NOS; NO-synthase, eNOS; endothelial NOS, nNOS; neuronal NOS, ONH; optic nerve head, POAG; primary open angle glaucoma, RNS; reactive nitrogen species, RGC; retinal ganglion cell, SC; Schlemm's canal, Tregs; regulatory T- cells, *Vac*A; Vacuolating cytotoxin A

CONFLICT OF INTEREST

None declared

FINANCIAL SUPPORT

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

INTRODUCTION

Nitric oxide (NO), a small (30 Da) anion radical, is expressed ubiquitously. It is involved in many physiological and pathophysiological pathways. As a gasotransmitter, it is a principal modulatory molecule ~~s~~ involved in mammalian biology, especially in the nervous, cardiovascular, and immune systems[1–6]. NO plays a physiological role in neuronal cell signaling. However, its overproduction induces neuronal energy compromise resulting in diverse neurodegenerative pathologies. NO is critical in the various functioning of the central nervous system (CNS). Toxic beyond certain levels, it contributes to pathophysiology in the context of inflammatory disorders. It also affects aging and neurodegeneration in the CNS[7–9].

NO-synthase (NOS), an intracellular cytochrome P450 enzyme, mediates NO production by catalyzing L-arginine to L-hydroxyarginine and further to NO and citrulline transformation[10]. NOS is divided into three NOS archetypal isoforms: (1) neuronal NOS (nNOS or NOS-1); (2) inducible NOS (iNOS or NOS-2); and (3) endothelial NOS (eNOS or NOS-3). Each isoform is associated with a set of characteristics and expression patterns[11,12]. nNOS and eNOS, are constitutively expressed; the third is induced by specific stimulants. nNOS, as the etymology reveals, is primarily found in the nervous system. It is essential for neuronal signaling by involvement in neurotransmission in astrocytes and neurons. The endothelial localization of eNOS is important for vasodilation and blood pressure control. The release of NO from eNOS under basal conditions regulates: organ blood flow distribution in the cardiovascular and renal system; inhibition of platelet aggregation, platelet and leukocyte adhesion and smooth muscle cell proliferation at the vascular level; and induction of natriuresis and diuresis in the kidneys[12,13]. Intracellularly, eNOS is detected in the Golgi complex, plasma membrane. It is attached to the mitochondrial membrane in various concentrations[6,14].

In contrast, iNOS is not constantly present in cells. It is only (over-) expressed when the cell is induced or stimulated, characteristically, by proinflammatory cytokines and/or bacterial lipopolysaccharide (LPS)[15,16]. In addition, it is overexpressed in response to different inflammatory stimuli. Nanomolar amounts of NO are generated by nNOS and eNOS for short periods of time (seconds to minutes) in a calcium/calmodulin (CaM)-dependent manner[3,16]. In contrast, upon triggered, iNOS induces substantial and delayed amounts of NO (micromolar range) up to several hours until the enzyme's degradation[17]. The produced amount of NO enhances the defense against invading pathogens. Therefore, it is critical for the inflammatory response and

the innate immune system. An inappropriately excessive NO concentration or dysregulation of iNOS can lead to toxic effects and is linked with a variety of diseases[15]. The dual activity of iNOS-related NO (beneficial vs. detrimental) is highly concentration-dependent. Therefore, regulation of its production is important for both maintaining its proper physiological functions and controlling its deleterious effects.

NO's neutral chemical affinity, stability and size allows its unimpeded diffusion through and beside cells using heme scavenging proteins like hemoglobin as buffers[6,18]. The initial, non-selective effect of NO-induced biochemical cascade on target cells is the activation of soluble guanyl cyclase to produce cyclic guanosine monophosphate (cGMP)[19]. More specifically, NO orchestrates a plethora of biological operations in main cellular compartments (i.e. nucleus, cytoplasm, mitochondria and cellular membrane) with an impact on overall cellular function, such as the inhibition of the mentioned platelet and neutrophil aggregation, relaxation of smooth muscles and regulation of the endothelial permeability[6,20,21]. In neural tissue it serves as a protective Janus molecule against degeneration and cellular death. In high concentration it induces reactive oxygen species (ROS) production and cytotoxicity[4,22,23]. NO becomes toxic after undergoing oxidation-reduction reactions with ROS to create noxious molecules recognized as reactive nitrogen species (RNS). The term "nitrosative stress" has been introduced to describe the cellular damage/toxicity owing to NO and RNS overload[24,25]. Excess of NO and/or the creation of RNS (e.g., peroxynitrite [$ONOO^-$], damages mitochondrial functioning. This, in combination with nuclear events, contributes to the neuronal cell metabolism disturbance and survival, promoting the pathogenesis of many neurodegenerative diseases[14]. $ONOO^-$ mediates most of the toxic actions of NO.

It is the microenvironment and its alterations that affect NO's formation, concentration, half-life and final action, whether subsidiary or harmful[26,27]. Normally, low

concentrations and a transient generation of NO regulate its actions to target cells. In contrast, inflammatory condition includes massive and perpetual NO secretion in high concentrations and nitrosative stress[27]. Although cGMP cascade remains the primary pathway of NO's cellular activity, secondary pathways have also been described; S-nitrosylation of proteins controls apoptosis, vascular resistance, and inflammation[28,29]. Specifically, NO's predominant function is mediated via activation of the mentioned cyclic guanyl cyclase. This signals a pathway and S-nitrosylation of various proteins being implicated in cellular homeostasis and in mitochondrial biology. Examples of NO-related mediators triggering neuronal cellular death involved in several neurodegenerative diseases include[11,14]:

- Increased mitochondrial fragmentation and autophagy via S-nitrosylation of important proteins like dynamin-related protein 1 and Parkin/PINK1 (protein phosphatase and tensin homolog-induced kinase 1) complex
- Formation of $ONOO^-$ involved in mitochondrial permeability transition and induction of nuclear toxicity with consequent release of apoptosis-inducing factor from mitochondria
- Activation of parthanatos
- Negative consequences on electron transport chain
- Glutamate neurotoxicity
- Changes in the mitochondrial metabolic pathways such as Krebs and urea cycles.

Glaucoma, a neurodegenerative disease, is characterized by progressive retinal ganglion cell (RGC) apoptotic death. This results in a *cupping* of the optic disk and blindness[30,31]. Ocular physiology sustains a perpetual and indestructible circle of

aqueous humor (AH) production by the ciliary body -the anterior portion of the uveal tract located between the iris and the choroid- passage from posterior chamber to the anterior chamber through the pupil. Drainage occurs at the anterior chamber angle through the human trabecular meshwork (H-TM) and Schlemm´s canal (SC), intrascleral channels, and episcleral and conjunctival veins[30,32]. Although complex pathophysiological aspects have not been completely illuminated, the dysregulation of the normal AH flow and intraocular pressure (IOP) is considered the main axis of glaucomatic disease[30]. AH outflow disruption, due to increased H-TM resistance or drainage pathway obstruction, conveys mechanical tension to the posterior ocular structures especially to the lamina cribrosa. This suppresses the axonal supply of neurotrophic factors within the RG cell axons. It is followed by a perpetual biochemical, inflammatory**,** and apoptotic degeneration of the RG cells[30]. The mechanical theory stresses the importance of direct IOP-related increased compression of the axonal fibers and support structures of the anterior optic nerve, with distortion of the lamina cribrosa plates and interruption of axoplasmic flow. This leads to RGC death[33]. Although this simplified cascade seems conceivable, it is only the tip of the iceberg. Molecular aspects and provocative events have not been clarified. Microcirculation disorders, altered immunity, excitotoxicity, and oxidative stress are considered to be involved[30].

Focus on NO-related glaucoma pathophysiology, a potential physiologic target of NO, as vasodilator, is the H-TM being strictly controlled by the balance between vascular dilators and constrictors(the endothelin [ET])[34]. NO is a cotransmitter of smooth muscle relaxation in the chamber angle. It may be implicated in the regulation of AH dynamics[35]. Both NO and ET appear to be regulators of ocular blood flow. Under normal settings, the vasodilating effect of NO is counterbalanced by the vasoconstricting effect of ET[36]. On the other hand, in the occurrence of free radicals,

NO creates toxic agents which provoke the TM metabolic states and modify its motility and function[36,37].

Proposed mechanisms of NO induced ocular homeostasis, after activation of cGMP cascade, include potassium kinetics' control, osmotic changes and the H-TM relaxation**.** These facilitate aqueous drainage and reduce IOP by regulating TM cells contractility and volume[38–40]. These physiological principles have raised the hypothesis that NO signaling malfunction could result in H-TM rigidity and increased IOP, which predisposes glaucoma. In this regard, permanent ocular injury could be a result of the vascular disharmony and dysregulation, thereby provoking an unstable perfusion pressure, transient ischemia**,** and reperfusion damage[41]. These mechanisms, although rationale and attractive, need further confirmation.

Regarding NO-connection with *Helicobacter pylori* infection (*Hp-I*), studies in patients with *Hp*-I revealed higher systemic and intraluminal levels of stable NO metabolites. This implies that *Hp* might induce iNOS generation expression[42,43]. The fundamental role of NO in glaucoma or other neurodegenerative disorders and its (over)-generation in patients with *Hp-I* has attracted the interest of recent studies attempting to elucidate the pathophysiological link between these conditions.

The present review aimed to imprint the role of NO in both glaucoma and *Hp*-I with a stimulating approach to motivate future studies attempting to investigate their potential relationship and pathophysiology, thereby introducing relative therapeutic strategies.

NO DOUBLE-EDGED SWORD: BENEFICIAL EFFECTS ON OCULAR PHYSIOLOGY

The range of systematic NO actions in vasodilation and intra- and inter-cellular permeability[44,45] reflects the mainstay of the beneficial role of NO within the eye, by promoting AH outflow. It affects H-TM and SC cell volume and increases AH drainage[39,40,46]. More specifically, H-TM consists of smooth muscle-like cells which dilate after NO's direct signaling[39,40] coordinates with the concomitant relaxation of smooth muscle cells around the intrascleral vessels. The post-trabecular, distal drainage resistance is reduced[45].

In normal conditions, a small quantity (in picomole levels) of NO is generated by interactions between L-arginine (as substrate) and nNOS and/or eNOS (as synthetic enzymes). NO may activate the mentioned guanyl cyclase to create the c-GMP. Consequently, c-GMP induces several biological responses, such as vasodilation, increase of ocular blood flow, decrease of IOP, and signal transduction of the nervous system[47–49].

Specifically, to chart the ophthalmic distribution of NO and understand its role to IOP regulation, NOS subtypes and ocular topography should be defined. eNOS is widely expressed in the SC cells, uveal vascular endothelium, H-TM, ciliary body and prelaminar region of the optic nerve[19,45,50–52]. Moreover, nNOS is primarily expressed in the inner retina, between the inner nuclear and the ganglion cell layers, with predominance in amacrine, bipolar and Müller cells, as well as the limbus, cornea, and lens[53–55]. On the other hand, iNOS -absent in physiological conditions- is suggested to prevail in H-TM, through macrophages after perfusion pressure elevation, and in iris via cytokine and endotoxin stimuli. This induces retinal ischemia, inflammation and excitotoxicity, in cornea, ciliary body, retinal pigment epithelial cells**,** and the lamina cribrosa astrocytes in established glaucoma[45,52,56,57]. Figure 1 illustrates the ocular locations of NOS subtypes.

eNOS is considered as the pivotal component regarding ocular protection against glaucoma. It facilitates mechanical decongestion of AH after its generation in H-TM by exerting physiological endothelial cell function[51,58]. The topical improvement of the blood flow (a substantial parameter of glaucoma development) in the optic nerve and the ciliary and retinal vessels is succeeded by the autocrine action of eNOS in the vascular endothelium, the main source of eNOS[51,52,59–63].

Regarding the protective role of NO in glaucoma, the aspect that the basal endogenous expression and release of NO maintain the normal ocular tone and IOP is supported by the results of human and animal model studies using NOS inhibitors, such as N-nitro-L-arginine methylester (L-NAME), N-nitro-L- arginine, and aminoguanidine [64,65]. In accordance to this, the ophthalmic artery the main ocular irrigation vessel, remains in a perpetual dilation as a consequence of a balanced antagonistic action of the mentioned vasoactive molecules, NO and ET[36,66]. More indirect credentials of the protective role of NO in glaucoma emanate from data, underlying the positive effect of NO in patients with primary open angle glaucoma (POAG), through relaxation of the H-TM, volume regulation, and increase of the permeability of SC. The latter effect is feasible after latanoprostene bunod administration, a NO-donating prostaglandin F receptor agonist activated in the anterior eye[11,59,67–69]. Additionally, L-arginine and sepiapterin, through their roles as catalytic agents in NO generation, reduce as antioxidants the concentration of ROS, such as superoxide or peroxynitrite. This protects H-TM from injury[70].

Evidence from studies on animal models of RGC injury, revealed that Nipradilol offers an additional neuroprotection related to the NO by this agent[55,71]. Specifically, Nipradilol [3,4-dihydro-8-(2-hydroxy-3-isopropylamino)-propoxy-3-nitroxy-2H-1-benzopyran; molecular weight: 326.35], is registered both as a systemic hypotensive and an anti-glaucoma agent. It exhibits nonselective b-receptor and selective a-receptor

blocking properties with a NO donor action[72]. The mechanism of its ocular hypotensive effects involves inhibition of AH production owing to its b-blocking action and increased uveoscleral outflow due to the action of a-blocking agents. Nipradilol has vasodilator activity owing to NO release from its nitroxyl moiety. NO liberated from the Nipradilol exhibits a nitroglycerine-like vasodilatory action of increased retinal blood flow and optic nerve head (ONH) circulation in rabbits[72]. NO at low concentration could mediate the effect of Nipradilol in promoting axonal regeneration of RGCs in adult cats[72]. Specifically, the protective effect of Nipradilol in RGCs appears to be mediated by S-nitrosylation of antioxidative-related Keap1 protein due to its NO-donating effect. It also has been reported that Nipradilol promoted axon outgrowth in cat RGCs. However, whether NO/S-nitrosylation signaling for axonal regeneration is unclear[72].

Regarding reduced cornea penetration, short duration of efficacy and limited therapeutic index of most NO donors impede their clinical applications in glaucoma therapy. Recent studies reported a novel NO-donor delivery system[73]. This method is based on mesoporous silica nanoparticles (MSNs), which may overcome the aforementioned problems and deliver the NO-donating drug, sodium nitroprusside (SNP) to the target tissues of TM and SC[73]. In this respect, it has been proposed that SNP-MSNs delivery system can induce more exogenous NO and maintain higher NO levels in animal eye models. This may lengthen the period of IOP decrease appearing to be promising for regulating IOP in patients with POAG and ocular hypertension. Likewise, this system may support the development of a new generation of nanomedicine for diverse clinic applications including subcutaneous and nasal cavity drug delivery[73]. However, skepticism about possible long-term NO toxic overload might limit their therapeutic application and thus further studies are warranted.

NO DOUBLE-EDGED SWORD: A CAUSE OF OCULAR PATHOLOGY

In abnormal conditions large amounts of NO (in nanomole levels) are produced by iNOS from L-arginine. NO further oxidizes into NO_2^-, nitrite, peroxynitrite and free radicals to interact with several molecules (i.e. thiols, iron-sulfur centers of several enzymes, cytochrome oxidase or glycolytic enzymes). This leads to the modification of biological functions of cells, DNA injury, neurotoxicity, optic nerve degeneration, and numerous eye diseases, including glaucoma[48,49,74]. Evidence of abnormal NO production in glaucoma is also supported by genetic data indicating consistent associations between eNOS-related polymorphisms and POAG[75]. Increased levels of NO to twice normal values were shown in rat retinas with induced glaucoma associated with apoptosis and necrosis of RGCs[76,77]. Additional data support that glaucoma could occur due to NO neurotoxic effects at the ONH and retina, leading to a vicious circle after ONH degeneration and visual field loss[78,79]. This appears to be triggered by factors like ocular inflammation, LPS, namely a Gram negative endotoxin, or pro-inflammatory cytokines[47–49].

An attractive approach regarding NO's harmful role in glaucoma concerns its uncontrolled and unstable ocular concentration. Abnormal effects by NO could be caused by any quantitative imbalance both increasing and decreasing. Indeed, both underproduction and overproduction of NO in eye tissues can occur, leading to ocular malfunction and diseases, respectively. Therefore, ocular diseases could be treated by making up NO deficiency through NO donors and NO precursors (substrate of NO synthases) or reducing overproduction of NO through inhibition of iNOS activity.

In case of infections, high NO concentration is helpful due to its antimicrobial effect.

However, it can trigger, as a proinflammatory signaling molecule, the induction of adverse effects[14,55]. In this regard, studies have demonstrated that exogenous administration of direct NO donors or N-methyl-D-aspartate type (NMDA) could be responsible for delayed glaucomatous retinal damage, cellular rarefaction in the ganglion cell layer, and thinning of the inner plexiform layer[80]. The mechanism is probably mediated via activation of NMDA membrane receptors and indirect production of a large amount of NO. It may be inhibited by NO trammeling agents, NMDA receptor antagonists or nitro-L- arginine methylester treatment[80,81].

On the other hand, NO absence after endothelial damage, eNOS genetic deficits, or NO signaling abnormalities spoils the vasoactive balance with ET, thus increasing nonselectively the vascular and tissue resistance and contraction[10]. In this respect, glutamate is a neurotransmitter, the physiologic role of which is to transmit synaptic pulses. In pathologic states, it stimulates target cells, causing death (or excitotoxicity)[82]. Excitotoxicity induced by glutamate via NMDA receptors produces large amounts of NO. This appears to play an essential role in the neuronal loss in glaucomatous neuropathy[83]. Specifically, glutamate-induced excitotoxicity has been a common underlying mechanism in diverse neurological disorders, including glaucoma[84]. The damaging glutamate effect on RGCs has been established by exposing the retina to high glutamate concentrations *in vitro*[85] and *in vivo*[86]. This effect of glutamate on RGCs occurs via interaction with the mentioned glutamate receptors. While excitatory receptors are copious in RGCs, under normal circumstances homeostatic mechanisms inhibit overexpression of the receptors[86,87]. Excessive accumulation of glutamate results in overstimulation of NMDA receptors. In turn, it induces intracellular calcium influx. The resulted complex cascade activation attacks cell components and produces ROS[88] and apoptosis[89,90]. This is also involved in glaucomatous pathology[91].

Supporting data from experimental models indicate that there are augmented intraocular glutamate levels following acute IOP elevation[92]. Moreover, evidence from some experimental glaucoma models and glaucomatous human eyes indicate high glutamate concentrations in the vitreous[93]. However, other studies show normal levels of glutamate in the vitreous of experimental glaucoma[94].

Additional experimental data indicate the presence of ET-1 and high levels of NO in AH and vitreous in spontaneous glaucoma whereas the changes in glutamate levels are varied[95]. It is important to note that the peak stimulus for NO release is the activation of NMDA receptors by glutamate, whereas glutamate neurotoxicity is mediated, at least partly, by NO and mitochondrial damage. This signifies the connection of NO pathways with glutamate neurotoxicity[96]. Based on the evidence, inhibition of glutamate activity by modulation of NMDA-type receptors may be an essential strategy for neuroprotection[97].

NO excess leads to oxidative reactions with ROS, resulting in the mentioned RNS production[98–100]. This is usually caused by conditions like tissue ischemia-reperfusion injury, inflammation and sepsis[101]. Normal antioxidant clearance mechanisms neutralize low concentrations of RNS, such as peroxynitrite, whereas, upon plethoric, these radicals use essential cellular molecules as substrate to react (i.e. as proteins, cysteine, and glutathione). This provides a stable and extended deposit of NO after S-nitrosylation and mediating nitrosative stress and biological diversion[98].

In this respect, nitrosative stress, expressed by peroxynitrite activity, has been suggested as an important risk factor for glaucoma[56]. The mainstay of its chemical actions, in case of insufficient clearance, is the generation of 3-nitrotyrosine, which is additionally used as a diagnostic “footprint” of nitrosative stress detected with antinitrotyrosine antibodies[77,98,102,103], thus suspecting a long-term exposure to

peroxynitrite[104]. Alternatively, peroxynitrite induces the oxidation of sulfhydryl groups and lipids, prevents mitochondrial respiratory circle**,** and destabilizes cytoskeleton[105]. These chemical pathways have been recognized as pathogenetic to endothelial injury, neurotoxicity**,** and other systemic inflammatory disorders[101]. Additionally, through RNS, NO mediates the pathophysiology and cellular apoptosis in CNS injuries, ischemia, infections, and neurodegeneration[5,106]. Intraocular peroxynitrite anions, in an oxidative ocular milieu, disintegrate quickly to toxic hydroxide radicals after reaction with oxygen, copper, and iron[23]. This causes fundamental modifications in H-TM and RGC function and architecture, by reacting with cellular molecules[98,107]. Furthermore, except for formatting peroxinitrite, NO can directly induce apoptosis through protein and DNA degradation due to its free radical properties[58]. Regarding the aforementioned data, rodent studies revealed a protective role of NOS inhibitors against retinal hypoxia, apoptosis and NMDA-induced cell death, thus implying a neurotoxic role of NO[79,108]. Although this effect is more obvious in mice due to their anatomy, NO excess can act opposite to its normal decongestive facility on H-TM by relaxing the ciliary muscle[45,109–111].

The ocular anterior chamber, as the region of glaucoma development, possesses vascular characteristics and expresses the markers of endothelial dysfunction, as in other vascular conditions[112–114]. Endothelial cells' injury in H-TM consists of the vascular component of pathophysiology in glaucoma by provoking further molecular cascades and apoptosis of ganglion cells[112].

As stated, the main eNOS action is beneficial by regulating H-TM homeostasis. Nevertheless, under exposure to endotoxins, endothelial cells of diminutive vessels produce superoxide through eNOS[115]. eNOS dimers constitute the appropriate form to the normal enzymatic function, whereas its monomers – a result of L-arginine or

tetrahydrobiopterin penury- affect oxygen by generating hyperoxide[116]. Additionally, studies with glaucoma patients have revealed an impaired nicotin-amide adenine dinucleotide phosphate oxidase diaphorase concentration in the conventional outflow system and mutations in the eNOS gene, such as eNOS rs2070744 polymorphism associated with normal tension glaucoma[10,117,118]. Likewise, eNOS overproduction in iris vessels and decline in H-TM have been revealed in patients with POAG and predicts worse outcomes[58,62].

In contrast to the generation and activity of eNOS under physiological conditions, iNOS is regulated by cytokines and bacterial signaling molecules in immune reactions against infections, inflammation, and tissue hypoxia. It induces an overwhelming and perpetual NO formation in macrophages and endothelial cells[3,11,20,26]. Cellular and extra-cellular stress stimulates a plethora of regulative inflammatory molecules, such as the nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB) and mitogen-activated protein kinases respectively, which are both responsible for iNOS triggering[17,119].

- Inflammatory micro-environment, through tumor necrosis factor (TNF)-α and other signaling molecules, activates the cytosolic inert NF-κB and induces its translocation to the nucleus and affixture on iNOS gene promoter, thus resulting to its activation and transcription[119,120].
- Activated NF-κB enters the nucleus to provoke transcription of genes that mediate varied cellular events, including immunity, inflammatory process, proliferation, apoptosis, and cellular senescence[31].
- NF-κB activation is a frequent pathological pathway in several disorders characterized by inflammation, including glaucoma[31].
- NF-κB signaling is activated in response to elevated IOP, vascular disorders, and oxidative stress[121].

- NF-κB activation was detected in human glaucoma ONH astrocytes and experimental animal models[122,123].
- Inhibition of NF-κB may decrease inflammatory processes and protect RGCs[121].

Studies in patients with neurodegenerative disorders also suggest a pathogenetic contribution of iNOS expression[11,124]. Likewise, some data indicate that an IOP raise induces iNOS generation, thereby strengthening the hypothesis of the vicious circle in the relationship between NO and glaucoma**.** The astrocytes of the ONH express the iNOS *in vivo* among glaucoma patients and glial cells *in vitro* under mechanical and hypoxic stress[125]. Moreover, studies with rat models of glaucoma support the theory that pressure mediated degeneration in glaucoma may be triggered by iNOS overexpression by inducing RNS neurotoxicity. The latter is implied by the beneficial outcomes of selective inhibitors of iNOS, such as aminoguanidine and the enhanced NO level following the elevation of the pressure gradient over the H-TM[58,125,126].

The inadequate perfusion and oxygenation of retina induces iNOS overexpression and NO generation. This results in cytotoxicity, apoptosis, axonal damage and visual field changes[56]. Patients with POAG exhibit an elevated concentration of nitrotyrosine and an analogous deficit of visual fields to the iNOS expression and activity in the H-TM[60]. Furthermore, multifocal ocular location of iNOS has been disclosed in glaucoma patients astrocytes of the ONH, microglia, endothelial cells of iris capillaries, AH, and H-TM[60,61]. Once iNOS production is induced in astrocytes and microglia, as a result of inflammation and stress related signaling molecules, a large amount of NO is generated. It demolishes neural cells through apoptosis and parthanatos[11]. As S-nitrosylation of cellular functional proteins occurs, a cascade triggers tumor suppressor p53 production and results in p53-dependent apoptotic cell death. Moreover, the NO-excess dependent

peroxynitrite acts directly and destructively on DNA**,** thus promoting parthanatos induction[11].

Important to note, the crucial role of NO toxicity as the initiator of $ONOO^-$ mediated parthanatos in a variety of neurologic conditions[14]. Parthanatos (derived from *thanatos* [Greek *θάνατος*] the personification of death in Greek mythology) is characterized by poly (ADP-ribose) polymerase-1 (PARP-1), a DNA damage-sensing enzyme, activation, PAR translocation from the nucleus to the mitochondria that ultimately results in mitochondrial depolarization, and large-scale DNA fragmentation, the final step in the execution of parthanatos**.** NO kills neurons via parhanatos[127], playing an important role in several neurodegenerative disorders[128].

Beyond parthanatos and mitochondrial permeability transition, other mechanisms involved in neuronal death include intrinsic and extrinsic pathways of apoptotic process, autophagy, phagoptosis (phagocytosis), oncosis, necroptosis, ferroptosis, sarmoptosis, autosis, autolysis, paraptosis, and pyroptosis[129]. NO has been implicated in the aforementioned mechanisms[14,130–135]. Figure 2 charts the pathways related to NO concentration fluctuations.

Hp IN NO-RELATED GLAUCOME PATHOPHYSIOLOGY

Hp selectively infects and colonizes mostly the gastric epithelium, oral cavity, and intestinal epithelium. It is strongly involved in the development of pathologies, particularly upper or lower gastrointestinal tracts and a variety of systemic disorders and neurodegenerative diseases like Alzheimer's disease (AD) or glaucoma[136–138]. Behaving as microbiota, *Hp* and its virulent products induce lasting chronic gastrointestinal inflammatory processes and systemic inflammatory reactions by

orchestrating and modifying the character of immune system responses. Therefore, it contributes to the pathophysiology of gastrointestinal and systemic disorders[136,138–140].

In this respect, beyond gastrointestinal pathologies, active *Hp-I* induces humoral and cellular immune responses. Owing to the sharing of homologous epitopes to the host antigens (molecular mimicry), it cross-reacts with components of peripheral and brain nerves contributing and potentially perpetuating neural tissue damage observed in diseases like Guillain-Barré syndrome (GBS) and AD[141–143]. Molecular mimicry may also play a role in glaucoma pathophysiology[144].

Regarding *Hp*-related iNOS, several studies have reported iNOS increased production following *Hp-I* in both humans and animals. The up-regulated iNOS production results in an increase of NO production. This may lead to the increase of DNA damage and apoptosis. In this respect, classification of iNOS expression in the gastric mucosa could be used clinically to identify patients with a high risk for gastric malignancy. The host will attempt to terminate the infection by activating the mucosal generation of the cytotoxic RNS. In the extracellular space, NO released from macrophages can eliminate *Hp*[145]. An effective increased production of NO and oxy-radicals results in bacterium eradication. Nevertheless, *Hp* persists in the host, inducing a lasting chronic inflammatory reaction that could be deleterious to the host.

The point that *Hp* survives in this hostile environment (despite upregulation of iNOS) indicates that this pathogen has developed strategies to avoid NO-dependent eradication. Several studies have reported on the complexity of the *Hp* response to oxidative and nitrosative stress. Specific proteins transcripted by *Hp* enable the pathogen to cope with the damaging effects of NO. These systems are part of the pathogen protection against nitrosative stress[146]. Likewise, *Hp* may exhibit a direct effect on reduction in gastric mucosal blood flow by inhibiting NO production by iNOS,

thereby decreasing the vasodilatory and mast cell stabilizing action of NO[147]. Moreover, several *Hp* virulence agents contribute to its ability to evade the immune system and disrupt the host's cells.

One of the most known and well-studied *Hp* virulence agents, urease, modulates iNOS expression and NO release by inflammatory cells, hampering phagocytosis by gastric macrophages[148,149]. *Hp* urease induces NO and ROS production by endothelial cells[150]. *H*p is highly adapted to surviving in the gastric environment; a key adaptation is its virulence agent urease[151]. Urease is a crucial index in the capacity of *Hp* to avoid bactericidal activity of gastric acid acidity[152]. Moreover, glutamate appears to be an essential immunomodulator during *Hp-I* and *Hp*-related γ-glutamyltranspeptidase (an important enzyme for its colonization). It induces a tolegenic effect via activation of glutamate receptors[153]. Justino et al.[146] were the first to describe HP0013, an enzyme that catalyzes and degenerates NO, which reduces iNOS-dependent immunity against *Hp*. The degradation of NO in the gastric environment could function as a feedback signal to iNOS. This occurs during a plethora of (patho-) physiological processes, leading to an increased and unlimited production of NO systematically and intraocularly. It requires targeted evaluation to confirm.

Another studied virulence agent is cytotoxin-associated gene A (*Cag*A). This manipulates a subset of fundamental cell processes including adhesion, polarity, proliferation and motility, receptor mediated endocytosis, cytoskeletal rearrangements, inflammation, apoptosis, and cell cycle progression[154]. The presence of *Cag*A leads to an increased risk of gastric carcinogenesis[155]. Increased hydrogen peroxide levels and oxidative DNA damage have been described in *Cag*A-positive strains[156,157].

Vacuolating cytotoxin A (*Vac*A) is a third well-studied virulence agent. It is capable of inducing an influx of Ca^{2+} and generation of ROS that leads to NF-κB activation,

thereby increasing proinflammatory immune response[158]. Likewise, *Vac*A with additional *Hp* factors, acts as immune modulators that impair the activation and proliferation of a diversity of immune cells, including T lymphocytes. This suggests essential roles in immune suppression and evasion. Such diverse immune functions of *Vac*A accentuate its significant role in the tempering of an immune response, thereby facilitating *Hp* colonization to the gastric epithelium**,** as well as its potential immunomodulatory role on extragastric disorders[159]. In particular, the ability of *Vac*A-proficient *Hp* to skew T-cell responses toward regulatory T-cells (Tregs) indicates that *Hp-I* may contribute to pathogen immune escape by increasing gastric Treg response[159,160].

The inability of the host to eradicate the infection leads to a chronic inflammatory state with sustained oxidative stress within the tissue. ROS and RNS, induced by the immune and epithelial cells, damage the host cells and lead to DNA damage. *Hp* has evolved to evoke this damaging process while reducing the mentioned host's efforts to kill the pathogen. This long-lasting process with inflammation and oxidative stress can lead to gastric oncogenesis[161].

Beyond the topical level, studies have reported positive antibodies against *Hp* related *Vac*A both in serum and CSF of patients with the mentioned GBS syndrome and delayed F-wave latencies[142]. The target molecules of the specific antibody against *Vac*A in the CSF of GBS patients are probably associated with components of the peripheral nerve myelin, indicating a role in the immune responses of patients with the demyelinating form of GBS. *Hp* could induce the mentioned humoral and cellular immune responses that, due to molecular mimicry, cross-react with ganglioside surface components of peripheral nerves. Immune reactions against target epitopes in the surface membranes of Schwann cells or myelin lead to GBS[162]. Importantly, molecular

mimicry of host structures by the LPS's saccharide part of the gastrointestinal pathogens *Hp* and *Cambylobacer jejuni* are connected with the development of autoimmune sequelae observed in GBS[163]**.** *Cambylobacer jejuni* may also be involved in glaucoma pathophysiology[164].

Apart from NO, molecular mimicry**,** and cross-reactivity, *Hp-I* could influence the pathophysiology of GBS, brain neurodegenerative disorders and glaucoma via numerous other mechanisms, including the release of proinflammatory and vasoactive substances acting at a distance[136,142,165].

The authors reported a strong correlation between AH and serum specific anti-*Hp* IgG levels in patients with glaucoma. Therefore, the authors hypothesize that *Hp* antibodies may circulate in the bloodstream and enter the aqueous circulation via blood AH barrier. In AH, they could reach a substantial level sufficient to impact the development or progression of glaucoma[166]. Other studies also reported that autoimmune injury to the optic nerve could occur directly by autoantibodies or indirectly by system of a mimicked autoimmune response to a sensitizing antigen, damaging RGCs[167,168].

Hp-I and glaucoma share common pathophysiological events. Therefore, it is enough to consider the hypothesis of causal dependence between these conditions. As a relative example, cellular death in both gastric and ocular epithelium, namely in *Hp-I* and glaucoma pathologies , follows a pathway of mitochondrial oxidative stress[169]. Oxidative stress affects mitochondrial and endosomal membranes due to their interaction. However, the direct apoptotic pathway remains unclear[170].

Relative studies indicate that, beyond *Hp*-related RNS action that induce**s** gradual local tissue damage and gastric carcinogenesis[171], chronic *Hp-I*, characterized by fluctuated severity over time, induces alterations in NO concentration. A perpetual generation and action of RNS also contributes to an unstable perfusion pressure in ocular arteries,

transient ischemia and reperfusion damage[41]. Moreover, several strains of *Hp* stimulate vascular endothelial growth factor (VEGF) expression. This is related to worse gastric outcomes and may contribute to the ocular upregulation of NO by VEGF[172,173].

Once triggered[174], VEGF activates its endothelial receptors; and a downstream sequence increases the concentration of NO[10]**.** VEGF appears to be involved in glaucoma pathophysiology[175]. In this regard, the known vascular burden of *Hp-I* could affect the H-TM endothelium by the induction of the synthesis of tissue factors, cell-surface thrombin, platelet adherence, adhesion molecules, cytokines, and growth factors like VEGF, as well as inhibition of prostacyclin[176,177]. Proinflammatory cytokines involved during *Hp-I* enhance the possibility of vascular diseases. Eradication treatment could ameliorate endothelial injury [176,178].

The *Hp*-I induced iNOS up-regulation considered with the aforementioned role of iNOS in glaucoma could propose inductively iNOS as the "common denominator" of these conditions, though necessitating experimental evaluation.

Viewing the aforementioned data, the following *Hp*-related NO cascade, summarized in Figure 3, seems to take place in glaucoma pathophysiology:

Upon *Hp* gastric mucosa colonization, immune response is induced. Mucosal immigration of macrophages and lymphocytes producing iNOS also takes place[179]. Once lymphocytes, plasma cells and macrophages are chemotactically triggered, iNOS generation is performed, as deduced by elevated concentrations of NO metabolites in *Hp* infected patients[42,43,179,180]. Chronic antigenic stimuli, such as superficial bacterial LPS upon exposure to inflammatory cells regulates iNOS expression in order to generate NO and RNS. This is especially true with peroxynitrite, which attempts to eliminate the pathogen[149,179,180], although *Hp* could escape such attacks.

Persistent *Hp*-I could promote the expression of iNOS, producing large amounts of NO, inducing DNA damage and gastric pathologies[181–185]. *Hp*-I induces damage in the stomach and duodenum by releasing soluble factors. This activates inflammatory cells, including neutrophils, to produce cytotoxic mediators like NO and superoxide[186].

NO, a rapidly diffusing gas, appears to be a potent neurotoxin that may contribute to demyelination and neurodegeneration[187]. Specifically, NO is diffused effortlessly in the systematic circulation- as implied, for instance, by studies in animal models of cirrhosis and patients with advanced cirrhosis[188,189]. Therefore, is also diffused in the retina as a potential agent of RGC apoptosis in the glaucomatous eye[190].

Additionally, chronic inflammation by *Hp-I* releases inflammatory mediators being incriminated to disrupt the blood–retinal barrier (BRB)[191]. More specifically, *Hp*-induced cytotoxin VacA displays chemotactic activities to the bone marrow-derived mast cells (BMDMCs) and induces the BMDMCs to produce proinflammatory cytokines like TNF-α, interleukin-6 and other mediators like VEGF which disrupt the blood-brain barrier leading to the development of degenerative diseases, including AD and glaucoma[165].

Proinflammatory cytokines like TNF-α released in *Hp-I* to increase particularly the permeability of BRB, rendering the retina vulnerable to other harmful agents and contributing to the pathogenesis of glaucoma[192–194].

After BRB damage, vasoactive substances, such as ET and NO, are released and aggregated intraocularly, thereby causing vasoconstriction induced ischemia of the optic nerve and dysregulation of ophthalmic artery tone. This promotes increment of IOP[74,190,191].

Furthermore, the nasal and oral cavity are known reservoirs of *Hp* by which the bacterium translocates in the anterior surface of the eye, inducing eye pathologies[195–198]. Likewise, *Hp* may access the brain via the oral-nasal-olfactory pathway leading to neurodegeneration[192]. An original study[199] revealed *Hp* contamination and neutrophilic and lymphocytic infiltration in the H-TM and iris tissue of glaucomatous patients after surgical intervention. This suggests an *Hp-I* induced chronic inflammatory process leading to glaucomatous damage. The direct ocular colonization by *Hp* could induce local inflammation, iNOS generation and nitrosative stress. In this respect, some studies reported that augmented levels of toxic autoantibodies recognize glycosaminoglycans of the ONH in glaucoma patients could increase the susceptibility of the ONH to damage by altering the functional properties of the lamina cribrosa, its vasculature, or both [168]. Likewise, high title of the mentioned specific anti-*Hp* IgG potentially toxic antibodies in AH might also contribute to glaucoma severity[166]. These data, however, are warranted further research in depth.

Finally, regarding the mentioned parthanatos-related PAR (PAR-1/PAR-2), it also appears to play an essential role in *Hp* gastric mucosa adhesion and *Hp*-associated pathologies[200]; *Hp* may activate PARs, which contributes to the *Hp*-related pathological responses[201]. Specifically, PAR-2 is involved in neuroinflammation[202]. Moreover, PAR-1 is located in specific neuronal populations and glial cells and its activation could induce neurite retraction in olfactory neurons[203]. The latter might promote *Hp* access brain via nasal-olfactory pathway. This induces brain pathologies[192]. It is further supported by experimental studies that indicate that PAR-1 deficient mice are resistant to neuronal damage and neurologic deficits in a cerebral hypoxia/ischemia model[204,205].

CONCLUSION

NO acts bidirectionally in ocular milieu, thus provoking or protecting from glaucoma**.** The dominant NOS subtype and environmental conditions are the main regulators of its function. Additionally, chronic *Hp-I* irrigates a perpetual and fluctuating NO production, contributing to nitrosative stress. The epidemiological connection between *Hp-I* and glaucoma[166,206–209], combined with the indications of both remote and local regulation of ocular NO by *Hp*, encourage the elaboration of further studies to substantiate and elucidate the suggested relationship. However, the pathogenesis of glaucoma is multifactorial. Neither *Hp*-I nor NO consist of exclusive burdens of its generation.

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